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Differential regulation of gelatinases by transforming growth factor beta-1 in normal equine chondrocytes

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Summary

Objective: Cartilage destruction in osteoarthritis (OA) is associated with increased levels of several matrix metalloproteinases (MMPs), including the gelatinases MMP-2 and MMP-9. While increases in some MMPs may be destructive, up-regulation of others may result from increases in normal tissue turnover. The production of MMP-2 and MMP-9 by the anabolic transforming growth factor beta-1 (TGF-β1) in normal equine chondrocytes was investigated.

Design: Equine chondrocytes from clinically normal femoropatellar joints were maintained in alginate beads. After serum deprivation, cells were exposed to TGF-β1 at several concentrations for varying times. Activities of MMP-2 and MMP-9 were estimated by gelatin zymography, while mRNA for MMP-2, MMP-9 and collagen type II were detected using reverse transcription-polymerase chain reaction.

Results: Stimulation with TGF-β1 differentially regulated MMP-2 and MMP-9, with strong up-regulation of both MMP-9 mRNA and enzyme. Increases in MMP-9 enzyme were dose-dependent (0–49 h). There is some evidence suggesting a slight reduction in MMP-2 release following stimulation. Collagen type II mRNA was transiently increased following stimulation.

Conclusions: The different effects of TGF-β1 on MMP-2 and MMP-9 production by normal chondrocytes suggests different roles for these enzymes. The increases in both MMP-9 and collagen type II mRNA following stimulation may suggest a role for MMP-9 in tissue maintenance. Therefore, increased MMP-9 may be secondary to, as distinct from a cause of, cartilage damage. © 2001 OsteoArthritis Research Society International

Key words: Transforming growth factor beta-1, Chondrocytes, Gelatinases, Equine.

Introduction

Osteoarthritis (OA) is a degenerative condition resulting in loss of articular cartilage which, in severe cases, can result in full-thickness cartilage erosions with accompanying severe pain and disability. The mechanisms leading to cartilage loss are poorly understood, although much attention has focused on the involvement of members of the matrix metalloproteinase (MMP) family of zinc- and calcium-dependent endopeptidases[1]. Most extracellular matrix components are substrates for at least one MMP and there is now a consensus that these enzymes are essential for normal extracellular matrix remodeling and maintenance. Most MMPs are secreted as proenzymes which can be activated by removal of the propeptide region. Their activity is further regulated by non-covalent binding to one of a number of tissue inhibitors of metalloproteinases (TIMPs)[2].

Much evidence exists to show an association between OA and MMPs[3]. Increased levels of several MMPs have been found in OA joints, both in synovial fluid and in cartilage itself. These include collagenase-1 and -3 (MMP-1 and MMP-13 respectively)[4], stomelysin-1 (MMP-3)[5], membrane type-1 MMP (MT1-MMP)[6] and the gelatinases, MMP-9 and MMP-2[7,8]. The classification of MMP-2 and MMP-9 as gelatinases is arbitrary and both can degrade many substrates, including collagens type IV, V, VII, X and the cartilage-specific type XI[9,10]. They can also degrade aggrecan core protein *in vitro* although evidence suggests that a collagenase-type MMP is responsible for proteoglycan degradation within cartilage explants[11]. However, evidence that gelatinases may be significant in the degradation of collagen was demonstrated by Kozaci *et al.*[12] who showed that collagen loss from cartilage could be prevented by the use of a specific gelatinase MMP inhibitor.

The specific signals that control MMPs in cartilage are unknown. However, many growth factors have been identified that play central roles in regulating articular cartilage metabolism[13], including the production of some MMPs. Transforming growth factor beta (TGF-β) is perhaps the most widely studied of such factors. There are at least three isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3), which themselves comprise a subfamily within a wider group of structurally related polypeptide growth factors[14]. TGF-β is widely distributed and its effects differ greatly depending on tissue and cell type. With chondrocytes, differing TGF-β effects have been observed on both proteoglycan synthesis and proliferation depending on the culture system used[13]. TGF-β1 has been shown to up-regulate collagen synthesis in primary monolayer cultures of rabbit articular chondrocytes[15]. Differential regulation of collagenases following

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TGF-β1 stimulation has also recently been reported in human chondrocytes isolated from areas close to and distant from OA lesions[16]. MMP-1, MMP-8 and MMP-13 were all down-regulated by TGF-β1 in lesion-derived chondrocytes, while cells from areas distant from the lesion had decreased MMP-1 and MMP-8 but increased MMP-13.

In addition to increased MMP levels, OA cartilage also displays increased synthesis of both collagens and proteoglycans[17–19], showing that chondrocytes are capable of responding to damage. Despite such anabolic responses, net matrix loss still occurs and up-regulated MMP activities could account for such an outcome. The increased levels of some MMPs may be due to an abnormally rapid tissue turnover. While the normal roles of MMPs in cartilage are not well understood, it is possible that some may be produced concurrent with synthesis of matrix components, possibly acting to remove degraded or damaged material prior to the integration of the new material into undamaged matrix. Such repair and maintenance processes exist and gelatinases may be involved, given their ability to digest many matrix components, including in particular, denatured collagen.

The present study was directed towards studying the production of MMP-9 and MMP-2 in normal cultured equine chondrocytes following TGF-β1 stimulation. Production of collagen type II mRNA was used as an indicator of response by chondrocytes. Our results showed differential regulation of MMP-9 and MMP-2 by TGF-β1 stimulation of chondrocytes.

Method

MATERIALS

Full-thickness cartilage was obtained from clinically normal femoropatellar joints of horses killed for reasons other than joint disease. All samples were from skeletally mature horses aged between 3 and 7 years. Recombinant human TGF-β1 (rhTGF-β1), DMEM (with sodium pyruvate and 1 g/l glucose), fetal bovine serum (FBS), gentamicin, amphotericin B, bacterial collagenase (type II) and Trizol reagent were from Life Technologies (Paisley, Scotland, U.K.). Reagents for reverse transcription were also from Life Technologies, with the exception of RNase-free DNase I and recombinant human RNase inhibitor, which were from Promega (Southampton, U.K.). Low viscosity sodium alginate, bovine serum albumin (BSA; fraction V) and gelatin (from bovine skin) were from Sigma (Dorset, U.K.). Other chemicals were of reagent grade quality.

ALGINATE BEAD CHONDROCYTE CULTURES

Chondrocytes were isolated by overnight digestion of cartilage slices, pooled from left and right femoropatellar joints, with gentle stirring at 37°C, in serum-free DMEM containing 50 μg/ml gentamicin, 2.5 μg/ml amphotericin B and 0.1% w/v bacterial collagenase. Undigested material was allowed to settle before decanting the supernatant into centrifuge tubes. Cells were pelleted at 250 ***g*** for 10 min, pooled, and washed three times in 0.15 M NaCl. They were then resuspended in 10 ml 0.15 M NaCl, counted using a hemocytometer, and pelleted. Typically, between 10×10^6 and 15×10^6 cells were obtained. Encapsulation of cells in alginate was performed according to the method of Guo *et al.*[20] Cells were resuspended in 1.2% w/v sodium alginate in 0.15 M NaCl at a concentration of 3×10^6/ml. The suspension was expressed dropwise through a 21 gauge needle into a solution of 100 mM $CaCl_2$. The beads were incubated in this solution for 10–15 min to allow complete gelling of the alginate. They were then washed three times in 0.15 M NaCl and once in DMEM containing 10% FBS, gentamicin and amphotericin B ('complete medium'). All washes were for 10 min at room temperature with gentle mixing, in volumes of solutions at least 10 times that of the beads. The beads were then transferred to a 10×10 cm dish and 35 ml of complete medium added. Cultures were maintained at 37°C in a 5% CO_2-in-air atmosphere, with replacement of complete medium every 2 days.

STIMULATION OF CELLS WITH TGF-β1

Sufficient beads were transferred to a new dish 24 h prior to stimulation and 35 ml of serum-free medium added. To ensure adequate removal of serum factors within the beads themselves, cultures were incubated at 37°C for 3–4 h in this medium before replacing it with fresh serum-free medium for the remaining time prior to stimulation. Beads were transferred to a 24-well dish for stimulation. For each experiment, equal numbers of beads (between eight and 10) were used per well. Excess medium transferred with the beads was removed and 1 ml of serum-free DMEM containing 0.1% w/v bovine serum albumin and the appropriate concentration of rhTGF-β1 was added. Samples of medium were removed after the required period of incubation and the beads were then disrupted by repeated pipetting in 1.3 ml of Trizol reagent. Both media and Trizol samples were stored at −70°C until required.

PREPARATION OF TOTAL RNA AND cDNA

Total RNA was prepared from the Trizol samples according to the manufacturer's protocol. The RNA pellet was dissolved in water and the RNA concentration estimated by measurement of absorbance at 260 nm. Conversion of total RNA to single-stranded cDNA included reduction of contaminating genomic DNA by DNase I digestion by a modification of the method of Huang *et al.*[21]. After initial heat denaturation of the RNA along with 500 ng oligo(dT)$_{12-18}$, in a volume of 10 μl, at 70°C for 10 min followed by cooling on ice, first strand buffer, DTT, dNTPs, 20U RNase inhibitor and 1U RNase-free DNase I were added and incubated at 37°C for 50 min. DNase was inactivated by heating the mix to 75°C for 5 min, before addition of 200 U of Superscript II reverse transcriptase and an additional 20 U of RNase inhibitor. This mix was then incubated at 42°C for 50 min before inactivation of the reverse transcriptase by heating at 70°C for 15 min. Total reaction volume was 20 μl with final concentrations in 1X first-strand buffer of 5 mM DTT and 500 μM of each dNTP.

POLYMERASE CHAIN REACTION (PCR)

Primers for MMP-2 (5′-CAG ATC ACA TAC AGG ATC ATC G-3′ and 5′-GTC GGC GTT GCC ATA CTT CAC A-3′, product size 321 bp) were designed from a partial equine sequence cloned in our laboratories[22] (Genbank accession number AJ010314). Collagen type II primers (5′-AGA TCG AGA GCA TCC GCA GC-3′ and 5′-GCA GCC ATC CTT CAG GAC AG-3′, 521 bp) were designed from an equine sequence (U62528), while those for MMP-9 (5′-GAC ATY GTC ATC CAG TTT GG-3′ and 5′-GTA GAG TCT YTC RCT

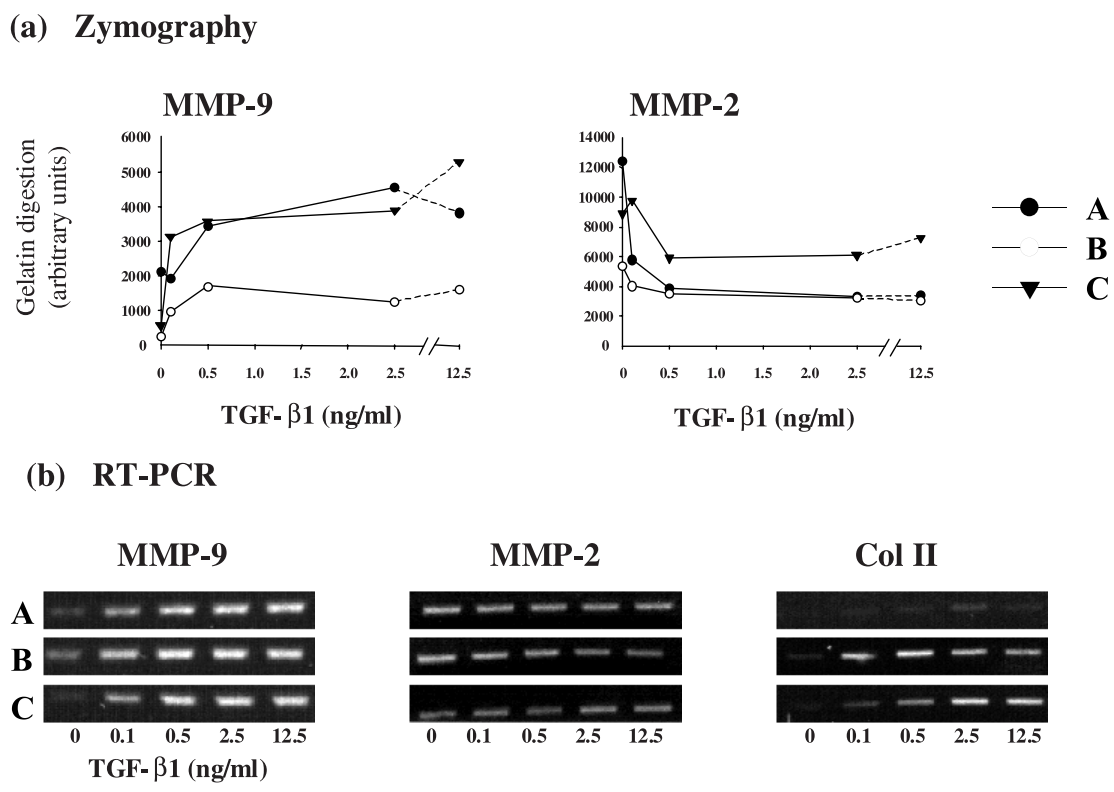

Fig. 1. Effects of TGF-β1 on chondrocyte production of MMP-9, MMP-2 and type II collagen. Alginate chondrocyte beads were cultured with concentrations of 0,0.1,0.5,2.5 and 12.5 ng/ml TGF-β1 for 24 h. (a) Gelatin zymography showing MMP-9 and MMP-2 enzyme activities in culture medium. Data represents combined values for the pro and active forms of each enzyme. (b) RT-PCR agarose gels showing MMP-9, MMP-2 and collagen type II mRNA.

RGG GC-3′, where Y=T or C and R=A or G, 339 bp) were designed from a consensus sequence derived from mouse, cow and human (X72795, X78324 and J05070 respectively). PCR reactions were carried out in 50 μl volumes containing 1 μl cDNA in a buffer of 1X Qiagen HotStarTaq PCR buffer (containing 1.5 mM $MgCl_2$), 200 μM of each dNTP, 0.5 μM of each primer and 1.25 U of HotStarTaq DNA polymerase. PCR amplification was preceded by an incubation at 95°C for 15 min to activate the polymerase, followed by 30–35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 15 min. Aliquots of PCR reactions were electrophoresed on 1.5% w/v agarose gels containing 0.5 μg/ml ethidium bromide, in TAE buffer (40 mM Tris-acetate; 1 mM EDTA, pH 8.3). Products were visualized on a U.V. transilluminator and photographed.

ZYMOGRAPHIC DETERMINATION OF MMP-2 AND MMP-9

Zymography was performed essentially according to the method of Kleiner and Stetler-Stevenson[23]. Briefly, 10 μl aliquots of sample, mixed with a 5X loading buffer (625 mM Tris.Hcl, pH 6.8; 5% SDS; 0.05% bromophenol blue; 10% v/v glycerol) and incubated at 37°C for 30 min, were electrophoresed on a 7.5% SDS-PAGE gel containing 1 mg/ml gelatin. Following electrophoresis, the gel was incubated in 2.5% v/v Triton X-100 for 1 h at room temperature, washed with water and incubated in a reactivation buffer (50 mM Tris.HCl; 50 mM $CaCl_2$; 0.05% v/v Brij 35; pH 7.8) at 37°C for 18 h before staining with a colloidal Coomassie blue stain. Gels were imaged by scanning at 400 dpi on a UMAX flatbed scanner. Estimates of band intensities were obtained by analysis of scanned images using the gel plotting macro supplied with the NIH Image software package, version 1.61 (available from the US National Institutes of Health, on the Internet at http://rsb.info.nih.gov/nih-image/).

Results

We investigated the effects of rhTGF-β1, on the production of MMP-2 and MMP-9 and on the mRNA levels of MMP-2, MMP-9 and type II collagen, by normal equine chondrocytes cultured in alginate. Chondrocytes were embedded in alginate directly after their release from cartilage by collagenase digestion and maintained by medium replacement for periods of between 2 and 6 weeks prior to their use in experiments. Since responses to growth factors often vary with time, we examined the time course, in addition to the dose-dependency, of the responses to TGF-β1.

DOSE-DEPENDENCY OF TGF-β1 REGULATION OF GELATINASES AND TYPE II COLLAGEN

Figure 1 shows the effects of a range of TGF-β1 concentrations (0, 0.1, 0.5, 2.5 and 12.5 ng/ml) on MMP-2 and MMP-9 enzyme released into the medium and determined by zymography [Fig. 1(a)]. Also shown are gels from RT-PCR reactions for MMP-2, MMP-9 and type II collagen [Fig. 1(b)]. Results are shown for three separate cultures

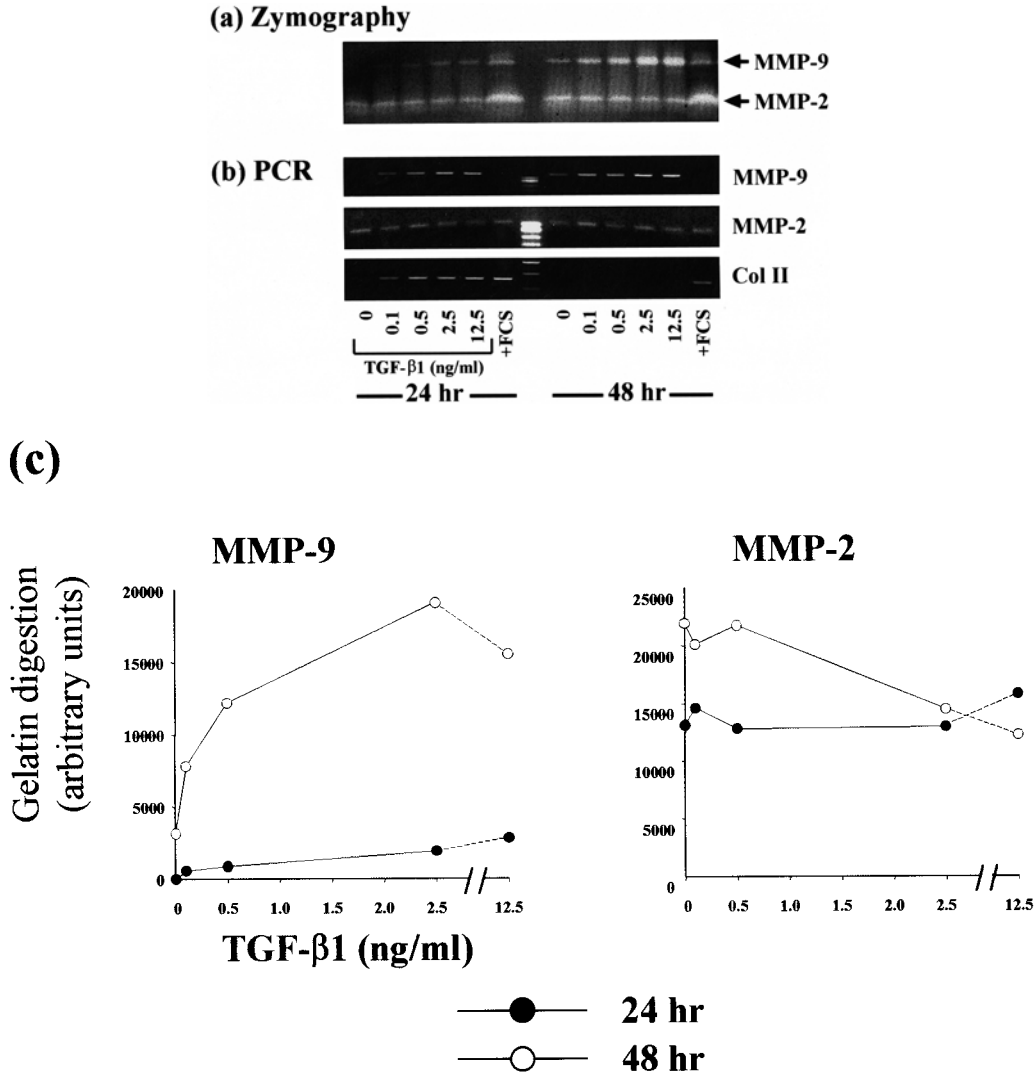

Fig. 2. The effects of the duration of exposure to TGF-β1 on MMP-9, MMP-2 and type II collagen production. Chondrocytes were cultured with TGF-β1 for 24 and 48 h. (a) Zymogram showing MMP-9 and MMP-2. (b) RT-PCR gels for MMP-9, MMP-2 and type II collagen mRNA. (c) Plots of MMP-9 and MMP-2 enzyme derived from the zymogram.

following 24 h of serum deprivation and 24 h of exposure to TGF-β1. Beads were transferred to multiwell dishes at the start of the stimulation period and fresh medium containing TGF-β1 added. Thus, zymographic results represent release of gelatinases from the beads into the culture medium over a 24 h period. Release of MMP-9 following stimulation was dose-dependently increased in all cultures, with a response observed at the lowest dose of 0.1 ng/ml in two cultures [Fig. 1(a); cultures B and C] and maximal effects apparent at doses between 0.5 and 12.5 ng/ml. MMP-2 release was decreased in all three cultures. The dose–response profile of this down-regulation was similar to that for MMP-9.

Messenger RNA levels for MMP-9, determined by RT-PCR, suggest an up-regulation in all cultures [Fig. 1(b)]. No consistent effect was observed with MMP-2 RT-PCR, although it is clearly important to note that non-quantitative RT-PCR, as used in this study, cannot give reliable indications of relatively small differences in mRNA levels. For cultures B and C in Fig. 1(b), levels of type II collagen mRNA were increased, with little up-regulation observed for culture A.

TIME-DEPENDENCY OF TGF-β1 REGULATION OF GELATINASES AND TYPE II COLLAGEN

The effects of an increased duration of exposure to TGF-β1 from 24 h to 48 h are shown in Fig. 2. There was a considerable increase in MMP-9 release between 24 and 48 h [Fig. 2(a), (c)], with a similar dose-dependency in each case. However, in contrast to the results shown in Fig. 1, no reduction in MMP-2 was apparent after 24 h in this experiment. Decreased release of MMP-2 was seen after 48 h. In unstimulated samples, there was an increase in the amount of each gelatinase released into the medium between 24 and 48 h [Fig. 2(c); points for zero TGF-β1]. This was particularly evident for MMP-9, for which the unstimulated 48 h medium sample contained more MMP-9 than the sample with most MMP-9 after 24 h (12.5 ng/ml). Thus, chondrocytes secrete both MMP-9 and MMP-2 during serum-deprivation. Levels of mRNA for MMP-9, MMP-2 and type II collagen are shown in Fig. 2(b). After both 24 and 48 h, there was an increase in MMP-9 mRNA levels, while for MMP-2 no consistent effect was observed, although band intensities did vary. Type II collagen mRNA

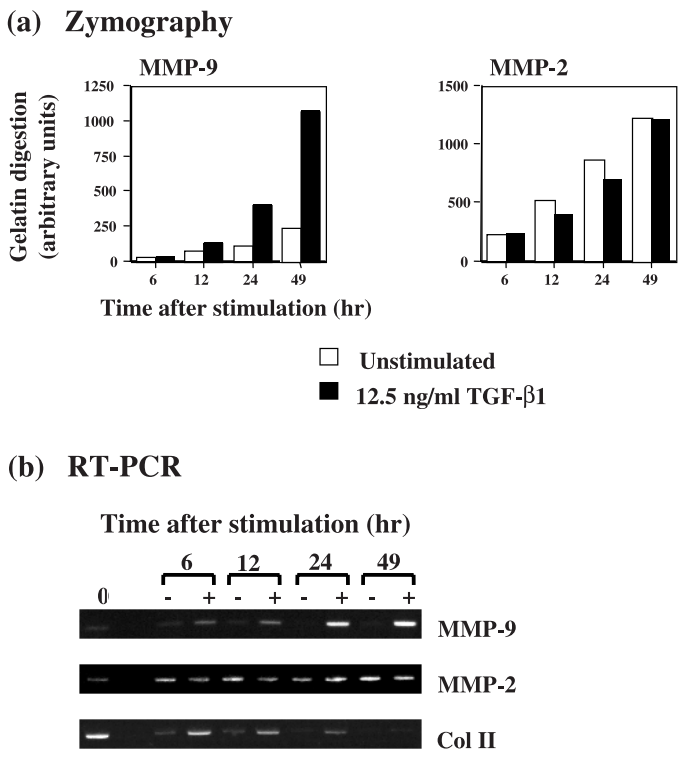

Fig. 3. Time course of induction of MMP-9, MMP-2 and type II collagen. Cells were cultured with 0 or 12.5 ng/ml TGF-β1 for the times indicated. (a) Histograms of MMP-9 and MMP-2 enzyme activities. (b) RT-PCR gels for MMP-9, MMP-2 and type II collagen.

was up-regulated following stimulation at all concentrations of TGF-β1 after 24 h. No signal was observed at any concentration after 48 h stimulation. Included in this experiment were cells cultured in DMEM containing FBS, but with no rhTGF-β1 [Fig. 2(a), (b); '+FCS' lanes]. These are equivalent to the unstimulated samples, but without serum deprivation. FBS contains high levels of gelatinases and consequently the zymographic findings cannot distinguish between FBS- and chondrocyte-derived activities. Figure 2(b) shows that serum-deprivation causes a reduction in the amount of type II collagen mRNA, but has no observable effect on MMP-9 or MMP-2 mRNA levels.

A time-course experiment (Fig. 3) examined MMP-9, MMP-2 and type II collagen at 6, 12, 24 and 49 h following exposure to either no growth factor or to 12.5 ng/ml TGF-β1, a dose which elicited near-maximal responses in other experiments. In unstimulated samples, levels of both MMP-9 and MMP-2 released into the medium increased with time [Fig. 3(a)]. Exposure to TGF-β1 caused an elevation in MMP-9 above unstimulated levels from 12 h onwards, while for MMP-2, stimulation caused a small inhibition of the increases seen with no TGF-β1. Stimulated MMP-9 RT-PCR band intensities increased with time, suggesting a time-dependent increase in mRNA levels, while relatively small differences were seen for MMP-2 [Fig. 3(b)]. Type II collagen mRNA levels were transiently increased following stimulation [Fig. 3(b); 0 h lane], suggesting that type II collagen levels were not down-regulated after the 24 h serum-deprivation which preceded stimulation.

Discussion

In this study we examined gelatinase regulation by TGF-β1 in alginate bead cultures of normal equine chondrocytes. The alginate culture system was chosen since previous work has shown that articular chondrocytes in alginate have a similar biochemical phenotype to those within intact cartilage, with respect to the types and proportions of collagens[24] and proteoglycans[25] produced. This is the case even for chondrocytes which were pre-cultured as monolayers, during which time expression of cartilage-specific genes is reduced[26]. Alginate cultures are also relatively stable, with retention of chondrocytic phenotype for periods of at least several months[27]. The alginate system, therefore, has many advantages over the use of chondrocyte monolayers which have many fibroblastic characteristics, including the production of large amounts of type I collagen, which is normally present in extremely low amounts in normal cartilage[26].

The key finding in this study is the differential regulation, by TGF-β1, of MMP-9 and MMP-2 production by normal equine chondrocytes. Much previous work on cartilage metabolism suggests an anabolic role for TGF-β stimulation of chondrocytes, including increased chondrocyte production of proteoglycans[28] and type II collagen[15]. Also, TGF-β1 antagonizes the catabolic effect that interleukin-1β has on cartilage matrix synthesis[29]. The anabolic effects of TGF-β1 were confirmed in this study, by the up-regulation of type II collagen mRNA expression. It is therefore paradoxical that TGF-β1 up-regulated production of MMP-9, an enzyme which clearly breaks down matrix components. One possible explanation is that the degradative function of MMP-9 operates in conjunction with matrix production as part of a matrix turnover and repair process. Such a role has not been identified for MMP-9. However, it is feasible that efficient degradation and removal of already compromised cartilage matrix, either before or concurrent with the laying down of new cartilage components, may form a part

of a matrix repair process. MMP-9 could be required for this function. There could be some other anabolic processes for which MMP-9 is required, but none has yet been identified.

Given that TGF-β1 stimulation causes a clear increase in the amount of mRNA for MMP-9, and in the production of enzyme, it seems likely that the increase in enzyme is due, at least in part, to increased synthesis by chondrocytes, as distinct from a simple release of stored intracellular enzyme. There was a much smaller and less repeatable inhibitory effect of TGF-β1 on MMP-2. It is unclear from the present work whether the slight reduction in MMP-2 release from beads into the culture medium is significant. Reductions in MMP-2 release may be due to a direct effect on production and secretion of this enzyme by chondrocytes. Our data were unable to provide evidence for such a direct effect. However, the results presented represent MMP-2 released from the alginate beads into tissue culture medium and an alternative explanation for decreased MMP-2 levels in the medium could be increased MMP-2 retention by the bead. This could occur, for example, as a consequence of MMP-2 binding to insoluble matrix components. Alternatively, there could be increased association of MMP-2 with TIMPs, predominantly TIMP-2. MMP-2:TIMP complexes would be substantially larger than the enzyme on its own and would consequently diffuse less readily out of the bead. Such retention could account for decreased MMP-2 levels in the medium of stimulated cultures despite similar amounts being produced by the chondrocytes in both stimulated and unstimulated cultures. Attempts to study MMP-2 and MMP-9 within beads by zymography demonstrated some retained gelatinases but due to severe smearing of bands, quantitative comparison between samples was impossible (data not shown). Physical entrapment of secreted products, such as gelatinase MMPs, could also occur within cartilage matrix, particularly when complexed to TIMPs. This is an area of research which justifies further study.

Since MMPs and TIMPs appear to act together to regulate much of the degradative activity in cartilage (and other extracellular matrices)[2], an increase in MMP-9 production by chondrocytes may not in itself lead to an increase in degradative activity. Additionally, most of the increased MMP-9 production was in the pro-MMP-9 form. Activation of this, by removal of the pro-peptide region is considered to be required for enzyme activity. One study on cartilage has shown an association between MMP-9 mRNA levels and areas of fibrillation on OA cartilage;[8] MMP-9 was virtually undetectable in normal cartilage. This suggests that MMP-9 enzyme may also be present in increased amounts in such regions of damaged matrix, although it gives no indication of its function.

The signals that cause changes in MMPs and other effectors within intact cartilage *in vivo* are generally unknown. In the case of MMP-9 up-regulation, our findings suggest that TGF-β1 must be considered a candidate growth factor capable of causing such a response. However, other factors also alter gelatinase production; in a study of normal human cartilage, interleukin-1α treatment caused similar changes to those found in our study, i.e. an increase in MMP-9 mRNA, with no change in MMP-2 mRNA[7]. Other factors, currently unidentified, but which may include physical as well as biochemical mediators, may also elicit similar responses. Indeed, since chondrocytes are highly differentiated and specialized cells, which function primarily to synthesize and maintain cartilage, it could be assumed that the effects of TGF-β1 shown in this study are therefore important in cartilage matrix integrity. The more we understand about the effects of growth factors, the better we will be able to explain how the processes are subverted in disease.

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